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GENOTYPING FANCONI ANEMIA PATIENTS FROM SERBIA REVEALS THREE NOVEL *FANCD2* VARIANTS

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Fanconi anemia is rare inherited disease characterized by wide spectrum of congenital anomalies, progressive pancytopenia, and predisposition to hematological malignancies and solid tumors. Molecular genetic analysis of mutations in *FANC* genes is of a great importance for diagnosis confirmation, prenatal and carrier testing, as well as for prediction of chemotherapy outcome and disease complications. In this study we performed screening of frequently affected regions of *FANCD2* gene for sequence variants in six unrelated FA-D2 patients in Serbia. This is the first molecular analysis of *FANCD2* gene in Serbian FA-D2 patients. A total of 10 sequence variants were detected, one in homozygous, and nine in heterozygous state. Two variants were found within exons, and eight within introns, in deep intronic regions. *In-silico* analysis showed that among all detected variants one exon variant and three intron variants might have impact on splicing mechanism. Heterozygous variants found in intron 3, c.206-246delG; exon 26, c.2396 C>A and intron 28, c.2715+573 C>T were not previously reported. *In-silico* analysis revealed that among them, two (intron 3, c.206-246 delG and exon 26, c.2396 C>A) could be novel disease-causing mutations. Many variants were found in more than one patient, including those unreported, indicating their possible ethnic association. Great number of variants in some patients suggests their non-random emergence in Fanconi anemia pathway.

Keywords: deep intronic variants, Fanconi anemia, *FANCD2* variants, splicing mechanism

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LIST OF ABBREVIATIONS

ΔCV – Delta Consensus Value; ALL – Acute Lymphoblastic Leukemia; BLAST – Basic Local Alignment Search Tool; BMF – Bone Marrow Failure; BRCA – Breast Cancer Gene; dbSNP – Database of Single Nucleotide Polymorphisms; DEB – Diepoxybutane; FA – Fanconi Anemia; FANCD2 – Fanconi Anemia Complementation group D2; HGVS – Human Genome Variation Society; HSF – Human Splicing Finder; LRG – Locus Reference Genomic; MMC – Mitomycin C; NCBI – National Center for Biotechnology Information; PAA – Polyacrylamide;
wt – Wild type

INTRODUCTION

Fanconi anemia (FA) is a rare inherited disease characterized by wide spectrum of congenital anomalies, progressive pancytopenia which often leads to bone marrow failure (BMF), and predisposition to hematological malignancies and solid tumors (SOULIER, 2011). Biallelic mutations in any of 19 up so far described FA genes, *FANCA* to *FANCT* (DONG *et al*,. 2015), which are a part of FA/BRCA DNA damage repair pathway lead to development of a corresponding complementation group (FA-A to FA-T complementation groups) of Fanconi anemia. In Serbia the most frequent complementation group is FA-D2 (VUJIC *et al.*, 2014), whereas in the world population it accounts between 1% and 3.3% (LEVITUS *et al*., 2004; KALB *et al*., 2007) of all FA patients.

The diagnostic approach for FA includes, besides physical examination, analysis of cellular sensitivity to DNA cross-linking agents such as diepoxybutane and mitomycin C (DEB and MMC) (AUERBACH, 2009) and determination of FA complementation group by immunoblotting (SHIMAMURA *et al*., 2002). Molecular analysis of germ-line mutation is of a great importance for diagnosis confirmation, prenatal and carrier testing, as well as for prediction of chemotherapy outcome (BORRIELLO *et al*., 2007). Additionally, even monoallelic mutations of a certain FA genes, such as *FANCD2*, are associated with the greater risk of developing hematological and solid tumors, such as T-cell acute lymphoblastic leukemia (ALL) and testicular seminoma (SMETSERS *et al*., 2012), which makes the molecular analysis important for evaluation of disease complications.

Mutation analysis can be difficult considering the size of FA genes and existence of pseudogenes. *FANCD2* gene (Locus Reference Genomic, LRG_306) is located on the short arm of the chromosome 3, 3p25.3 (TIMMERS *et al*., 2001). This gene is highly conserved in eukaryotic organisms, confirming its' important role in DNA damage repair. It contains 44 exons with the start codon localized in exon 2, and spans approximately 75.5 kb. Mutational analysis is hindered due to the existence of two known unprocessed pseudogenes, *FANCD2-P1* (NCBI Acc. No. NG_025673.2) located upstream of *FANCD2* and spanning 16 kb, and *FANCD2-P2* (NCBI Acc. No. XM_017030238.1) located downstream of *FANCD2* and spanning approximately 31.5 kb. Both pseudogenes show high percentage of sequence homology with the front (*FANCD2-P1*) and the middle portion (*FANCD2-P2*) of the *FANCD2* gene, which makes primer construction for specific gene amplification difficult to achieve. Therefore, despite the development of new technologies, there is limited number of reports regarding *FANCD2* mutations (KALB *et al*., 2007; AMEZIANE *et al*., 2012; KNIES *et al*., 2012; GILLE *et al*., 2012; CHANDRASEKHARAPPA *et al*., 2013; CHANG *et al*., 2014). Studies considering *FANCD2* mutations in FA-D2 patients showed hypomorphic nature of the mutations since the residual FANCD2 protein with a preserved function was always present as showed by immunoblotting (KALB *et al*., 2007). Additionally, Kalb and

coworkers in their extensive study showed that more than a half of mutated alleles reside within introns and result in aberrant splicing; many mutations are recurrent and have ethnic association.

The aim of this study was to screen frequently affected regions of *FANCD2* gene for sequence variants in FA-D2 patients from Serbia. This is the first molecular analysis of *FANCD2* gene in Serbian FA-D2 patients.

MATERIALS AND METHODS

Patients

A total of 6 unrelated FA-D2 patients were included in this study. Diagnosis of FA was previously confirmed by a positive DEB test, Western blot analysis and correction of cellular phenotype with particular *FANC* genes (AUERBACH, 2009; SHIMAMURA *et al*., 2002), and an assignment to FA-D2 complementation group was previously reported (JOKSIC *et al*., 2012; VUJIC *et al.*, 2014). This study was approved by The Ethical Committee of the Mother and Child Health Care Institute of Serbia „Dr Vukan Cupic".

DNA isolation, PCR and sequencing

Total DNA was isolated from patients' fibroblast cells using FlexiGene DNA Kit (Qiagen) according to the manufacturer instructions. The regions with frequently described mutations were screened for mutations. Amplification was performed using Phusion High Fidelity PCR Kit (Thermo Scientific). Primers used for the amplification of the target regions are shown in the Table 1. Primers for exons 10, 16, 26, 29 and 38 as well as for the amplicon I were used according to Kalb and coworkers (KALB *et al.*, 2007). Primers for exon 14, introns 3, 4, 12, 21 and 28 as well as for the amplicon II were designed using Primer3Plus software. Sequences of the gene and the pseudogenes were previously aligned (NCBI BLAST, ClustalW) and regions that do not share homology were identified for placing the primers. Since introns 12 and 21 have high homology with pseudogenes, two large superamplicones (amplicon I and II) were generated by primers positioned in flanking region (region without homology with pseudogenes; from this amplicons introns 12 and 21 were reamplified, respectively (Nested PCR)). Two pairs of primers were used for amplification of introns 3 and 28 due to their great length.

All primers were blasted against the whole genome for specificity. PCR products were purified using QIAquick kit (Qiagen) according to the manufacturer instructions and checked on the silver stained polyacrylamide (PAA) gels. The sequencing was performed on ABI 3730 capillary genetic analyzer, Applied Biosystems (Macrogen, Netherlands, Europe).

Sequence analysis

NCBI BLAST (*www.ncbi.nlm.nih.gov/BLAST/*) was used for homozygous variation detection, and FinchTV and DNA Tools Xplorer softwares were used for the heterozygous variation analysis. Designation of *FANCD2* variations was performed according to Human Genome Variation Society (HGVS) recommendations, Genome Reference Consortium Human Build 38 patch release 2 (GRCh38.p2 assembly), and Ensembl variation database. All found variants were checked in the currently available databases for reported mutations and single nucleotide polymorphisms (SNPs).

Table 1. FANCD2 exon, intron and amplicon primers

FANCD2 regions	Primer sequence (5'- 3')	Annealing temperature (^{0}C)
FANCD2 intron 3(1)	F: AGGAAGCAACCACTTTCCAA R: GACCTGCCATAACCTTAGCAA	60^0C
FANCD2 intron 3(2)	F: TGTTTTGGGGGAGCAGATTA R: GGGATAGGAAGGGTGTCTCC	56^0C
FANCD2 intron 4	F: AGACACAACCCCATGACTCTG R: GCAGACGCTCACAAGACAAA	60^0C
FANCD2 exon 10	F: GCCCAGCTCTGTTCAAACCA R: CATTACTCCCAAGGCAATGAC	65^0C
FANCD2 intron 12	F: CTGGACTGTGCCTACCCACT R: TCAGCGAAACACGTTACACC	60^0C
FANCD2 exon 14	F: GTTGCCAGATGGACACATTG R: GACCTGGCTTCTTTGACTGC	65^0C
FANCD2 exon 16	F: AGGGAGGAGAAGTCTGACATT R: TTCCCCTTCAGTGAGTTCCAA	65^0C
FANCD2 intron 21	F: GCAGATTGGAAAAGGGATGA R: CTACGAAGGCATCCTGGAAA	65^0C
FANCD2 exon 26	F: GACATCTCTCAGCTCTGGATA R: TCAGGGATATTGGCCTGAGAT	65^0C
FANCD2 intron 28(1)	F: ATGTGACCCTACGCCATCTC R: AGCTTAGGGGCCAGAATGAT	58^0C
FANCD2 intron 28(2)	F: TTCTGTAAGAGCCCAAGTTTCA R: ACCTCAATGTCCAGCTCTCG	60^0C
FANCD2 exon 29	F: CTTGGGCTAGAGGAAGTTGTT R: TCTCCTCAGTGTCACAGTGTT	65^0C
FANCD2 exon 38	F: GCACTGGTTGCTACATCTAAG R: AAGCCAGGACACTTGGTTTCT	65^0C
FANCD2 amplicon I	F: TGCTCTTCTCTGTCCCCAGA R: CATTGGGCGTGTATTCAGGG	58^0C
FANCD2 amplicon II	F: GCTTCTAGTCACTGTCAGTTCACCAG R: ACGTTGGCCAGAAAGTAATCTCAG	60^0C

In-silico analysis

FANCD2 variants, especially those found in intronic regions and not previously reported, were further evaluated for the potential effect on the splicing. Human splicing finder, HSF (*http://www.umd.be/HSF3/*) was used for the prediction of the intronic variants effects on the splicing mechanism. Pathogenicity of the exonic variants that lead to amino acid substitutions, was assessed via PolyPhen2 (*http://genetics.bwh.harvard.edu/pph2/*) program.

RESULTS

Of all regions of *FANCD2* gene that have been screened in patients, a total of 10 sequence variants were found. Table 2 summarizes all detected variants and a predicted consequence, if known, in the currently available databases. Most of the nucleotide changes were found at the heterozygous state, except for one found in all patients in intron 12 at homozygous

state (c.990-133 G>T), however this variant is commonly present in the healthy population, and has no clinical impact. *In-silico* analysis predicted that among all variants found, four (intron 3, c.206-246delG; intron 12, c.990-38 C>G; intron 28, c.2716-306 A>G; and exon 14, c.1122 A>G) might lead to the generation of the new splice sites. Heterozygous variants found in intron 3, c.206-246delG; exon 26, c.2396 C>A and intron 28, c.2715+573 C>T were not previously reported.

Table 2. Detected FANCD2 variants, frequencies and predicted effects

Region of FANCD2 gene	Genomic position (GRCh38.p2, Current assembly)	Human Genome Variation Society nomenclature	Frequency of allele in general population/ European population (HapMap)	Frequency of genotype in general population/ European population (HapMap)	Homozygous/ Heterozygous	Predicted effect on protein function	Case number	Ensembl variation database name (dbSNP)
Intron 3	**g.10034223 delG**	**c.206-246 delG**	-	-	Heterozygous	Not previously reported, creation of a new acceptor splicing site	1 2 4	-
Intron 12	g.10043351 G>T	c.990-133 G>T	0.952/0.913	0.089/0.171	Homozygous T/T	Intron variant	1 2 3 4 5 6	rs803335
Intron 12	g.10043446 C>G	c.990-38 C>G	0.212/0.139	0.284/0.235	Heterozygous C/G	Intron variant, creation of a new acceptor or donor splicing site	1 4	rs9809061
Exon 14	g.10043852 A>G	c.1122 A>G	?/0.034	?/0.067	Heterozygous A/G	Protein coding sinonimous variant, creation of a new donor splicing site	1 2 4 6	rs34046352
Intron 14	g.10043940 G>T	c.1134+76 G>T	0.300, small sample	0.600, small sample	Heterozygous G/T	Intron variant	1 2 4 6	rs35870071

Table 2. continued

Region of FANCD2 gene	Genomic position (GRCh38.p2, Current assembly)	Human Genome Variation Society nomenclature	Frequency of allele in general population/ European population (HapMap)	Frequency of genotype in general population/ European population (HapMap)	Homozygous/ Heterozygous	Predicted effect on protein function	Case number	Ensembl variation database name (dbSNP)
Intron 16	g.10048089 A>C	c.1413+38 A>C	0.179/0.139	0.272/0.235	Heterozygous A/C	Intron variant and non coding transcript variant (retained intron)	4 6	rs7615646
Intron 21	g.10064163 A>G	c.1948-193A>G	0.026/0.070	0.049/0.131	Heterozygous A/G	Intron variant and nonsense mediated decay transcript variant	1 2 5	rs2347585
Exon 26	**g.10067219 C>A**	**c.2396 C>A**	-	-	Heterozygous C/A	p.A799D	2 5	-
Intron 28	**g.10074250 C>T**	**c.2715+573 C>T**	-	-	Heterozygous C/T	Unknown, not previously reported	1 2 4 6	-
Intron 28	g.10074224 A>G	c.2716-306 A>G	0.001/0.002	0.002/0.004	Heterozygous A/G	Intron variant, creation of a new donor splicing site	2	rs192804303

*Bold variants are not found in the currently available databases.

The *Ensembl* Variation *database*

The Short Genetic Variations database (dbSNP) *(NCBI dbSNP database)*

Fanconi anemia mutation database

Exon variants

Two sequence variants in exons were found, heterozygous variant in exon 26, c.2396 C>A, and heterozygous variant in exon 14, c.1122 A>G whereas all other changes were detected within deep intronic regions.

Bioinformatics analysis of genomic variant in exon 26, c.2396 C>A (Figure 1) which was found in heterozygous state in two patients (cases 2 and 5) showed that this is probably damaging missense mutation p.A799D (PolyPhen2, score - 0.99). This variant has not been previously reported.

Genomic variant in exon 14, c.1122 A>G was found in four patients in heterozygous state (cases no 1, 2, 4 and 6). This variant was reported in dbSNP - rs34046352, and referred as "protein coding synonymous variant", but experimental data on its function was not available. *In-silico* analysis of this variant using HSF predicted activation of an exonic cryptic donor site with potential alteration of splicing (HSF scores: wild type (wt) – 40.32, mutant – 67.16, delta consensus value (ΔCV) – 66.57%).

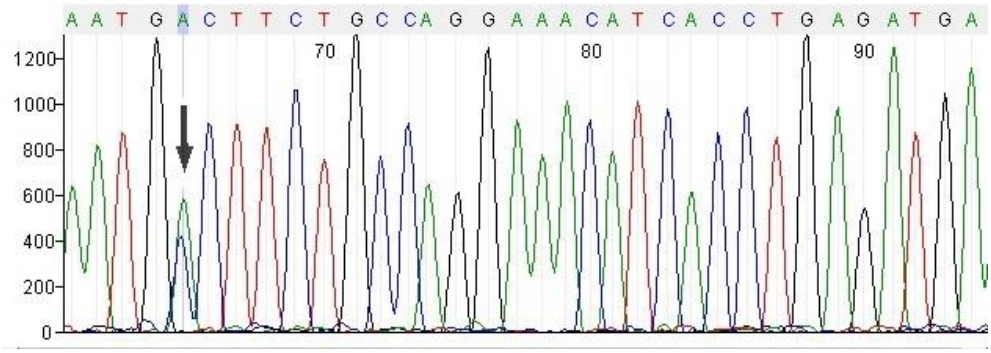

Figure 1. Sequencing chromatogram of *FANCD2* variant in exon 26, c.2396 C>A. Arrow indicates the presence of two peaks on the position c.2396 of *FANCD2* gene.

Intron variants

Eight sequence variants were detected within intronic regions, many of which have been reported in databases (Table 2). Among all found intronic variants, *in-silico* analysis showed that three variants (intron 3, c.206-246 delG; intron 12, c.990-38 C>G and intron 28, c.2716-306 A>G) might have impact on splicing mechanism.

Unreported variant in intron 3, c.206-246delG which is found in three patients (cases 1, 2 and 4) is predicted to generate a new acceptor splicing site (Figure 2), upstream to the physiological acceptor splice site to the exon 4, with a very high score, according to HSF (HSF scores: wt – 10.13, mutant – 77.24, ΔCV – 662.49%). Creation of a new acceptor splicing site was also predicted for the intronic variant in intron 12, c.990-38 C>G, found in two patients (cases 1 and 4), upstream to the physiological splice site to the exon 13 (HSF scores: wt – 56.61, mutant – 85.55, ΔCV – 51.12%). This variant was previously reported in dbSNP (rs9809061), however its effect was not assessed.

Variant detected in intron 28, c.2716-306 A>G is predicted to generate a new donor splicing site downstream to the physiological donor splice site (HSF scores: wt – 40.04, mutant – 66.87, ΔCV – 67.01%). Although reported in dbSNP - rs192804303, the presence of this variant is rather low in the world and European population - 0.001, and its functional significance is not assessed.

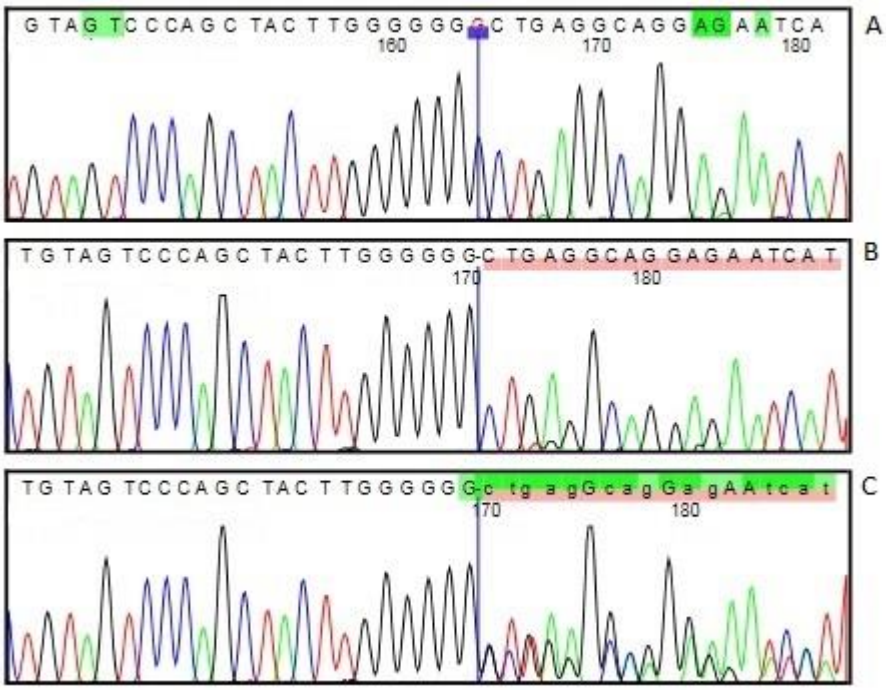

Figure 2. Sequencing chromatogram of *FANCD2* variants in intron 3, c.206-246delG. Blue vertical lines indicate position where the deletion has occurred; chromatogram of the control sample (A), heterozygous indel (B) and heterozygous deletion (C) in affected patient are presented. Double peaks after deletion can be observed (C).

Distribution of FANCD2 genomic variants among patients

As seen in Table 3, in patient 3 only one variant was detected (common variant in intron 12 in homozygous state, also detected in all other patients). In all other patients three or more variants were found, whereas in three patients (1, 2, and 4) up to eight variants were detected. According to the prediction results (mutant scores <70 were considered ambiguous and therefore irrelevant without further investigation), in patients 1, 2 and 4 two pathogenic variants were found.

In patient 5 only one pathogenic variant was found, whereas in patients 3 and 6 none of the genomic variants detected is predicted to be a disease causing.

In-silico analysis revealed that among three previously unreported variants, two (intron 3, c.206-246 delG and exon 26, c.2396 C>A) could be novel disease causing mutations.

Table 3. Unreported and reported FANCD2 variants in FA-D2 patients

	FANCD2 variants (according to Human Genome Variation Society nomenclature)		
Case number	Unreported variants	Variants with low frequencies <1%	Variants with frequencies >1%
1	c.206-246 delG	-	c.990-133 G>T
	c.2715+573 C>T		c.990-38 C>G
			c.1134+76 G>T
			c.1948-193A>G
			c.1122 A>G
2	c.206-246 delG		c.990-133 G>T
	c.2715+573 C>T	c.2716-306 A>G	c.1134+76 G>T
	c.2396 C>A		c.1948-193A>G
			c.1122 A>G
3	-	-	c.990-133 G>T
4	c.2715+573 C>T	-	c.990-133 G>T
	c.206-246 delG		c.990-38 C>G
			c.1134+76 G>T
			c.1413+38 A>C
			c.1122 A>G
5	c.2396 C>A	-	c.990-133 G>T
			c.1948-193A>G
6	c.2715+573 C>T	-	c.990-38 C>G c.1122 A>G
			c.1134+76 G>T
			c.1413+38 A>C

DISCUSSION

The analysis of particular *FANCD2* regions in FA-D2 patients in Serbian population revealed ten genomic variants, three of which have not been previously reported. One novel missense mutation found in two patients in exon 26, c.2396 C>A leads to the substitution of alanine with aspartic acid at the position 799 (p.A799D). According to the prediction program PolyPhen2, this mutation is pathogenic and could lead to partial degradation of protein. Another novel genomic variant c.206-246delG detected in intron 3 in three patients is predicted to affect splicing, i.e. creates a new acceptor splicing site 246 nt upstream to the physiological splicing site and may lead to intronic exonisation.

Additionally, among other detected variants which have already been reported to the dbSNP, prediction tools revealed that three could influence the splicing pattern of *FANCD2* gene.

Even though reported, the frequency of these variants is low in the population (Table 2), and their functional significance has not been assessed. Creation of an aberrant acceptor splicing site was estimated for the variant in intron 12, c.990-38 C>G, upstream to the physiological splicing site for the exon 13. Despite the fact that this variant is reported in the dbSNP (rs9809061), its pathogenicity has not been determined. It is important to mention that known SNPs, as well as synonymous mutations can also lead to the disease, and requires further investigation (CHANG *et al.*, 2014; CHANDRASEKHARAPPA *et al*., 2013), therefore they cannot be excluded as variants related to FA disease. Both of these variants (intron 3, c.206-246delG and intron 12, c.990-38 C>G) anticipated to create alternative splicing sites have high prediction scores for the mutant allele, scores below the threshold for the wt allele and high delta between the wt and the mutant CV (Table 4), suggesting great probability of creation of the splice sites in intronic regions where splicing normally doesn't occur. According to Desmet and coworkers in their detailed and comprehensive study including 83 intronic and 35 exonic mutations known to cause aberrant splicing, HSF was able to correctly predict the effects of these mutations with very high sensitivity, especially for intronic mutations with such high scores (DESMET *et el.*, 2009). Additionally, studies which included *in-silico* and functional analysis showed that HSF is the most reliable and informative prediction software (NASCIMBENI *et al*., 2010).

Two variants of unknown significance reported in the dbSNP were predicted to create alternative donor splicing sites, intron 28, c.2716-306 A>G and exon 14 c.1122 A>G (rs192804303 and rs34046352, respectively). The variant in intron 28 is found with very low frequency in the world and European population (0.001 and 0.002, respectively; data from 1000 genomes and HapMap project) and the effects of this variant are not known. Variant in exon 14 is reported as protein coding synonymous variant, but without experimental data of its pathogenicity. Nowadays it is known that synonymous and silent mutations can lead to modification of protein conformation and function, so despite once considered harmless, their importance in human diseases is widely accepted (CHAMARY *et al.*, 2006). Albeit both of these variants (intron 28, c.2716-306 A>G and exon 14 c.1122 A>G) were estimated to create a new donor splicing site by HSF, mutant scores were slightly above the threshold values, and are therefore considered ambiguous without further functional analysis.

Recent studies focused on identifying disease causing mutations in *FANCD2* gene pointed out the hypomorphic and heterogeneous nature of found mutations. Apparently, homozygous mutations in *FANCD2* are rather rare, but compound heterozygous mutations are mostly found. Furthermore, the great majority of reported mutations were found within intronic regions, near the exon-intron boundary or in deep intronic regions (KALB *et al.*, 2007; CHANDRASEKHARAPPA *et al.*, 2013; AMEZIANE *et al.*, 2012). Presence of missense mutation which leads to FANCD2 protein with altered activity and intronic mutations which can cause aberrant splicing is consistent with the fact that residual amount of FANCD2 is present in all FA-D2 patients.

Detection of same variants, which are predicted to be pathogenic, in several unrelated patients (Table 2), such as intron 3, c.206-246 delG in three patients, exon 14, c.1122 A>G, in four patients and exon 26, c.2396 C>A in two patients suggests that these variants could be population-specific and due to the founder effect of ancestral allele. Kalb and colleagues also found that some *FANCD2* mutations are highly specific for the patients' origin, such as splice mutation in intron 21, c.1948-16 T>G which is strongly related to the Turkish origin (KALB *et al.*, 2007). Association of specific mutations with the certain populations was also found in other FA genes (MADJUNKOVA

et al., 2014; TIPPING *et al*., 2001). Variants in intron 3, c.206-246 delG and exon 26, c.2396 C>A, found in this study, were not previously reported, even though large-scale studies on FA-D2 patients from multiple populations have been performed, indicating their possible linkage to the Serbian population.

Another interesting finding in our study is that in three patients (cases 1, 2 and 4), up to eight *FANCD2* variants were found, whether assessed to be pathogenic or not (Table 2 and 3). As proposed by Chang and colleagues (CHANG *et al*., 2014), high frequency of heterozygous variants suggests that mutations doesn't occur randomly, but might be linked and reflect the susceptibility of FA pathway, or in this case *FANCD2* gene. They found a great number of concomitant mutations in FA patients, and not only in one gene, but throughout the FA pathway genes, which implies that assignment to some FA complementation group doesn't exclude mutations in other *FANC* genes. Knies and colleagues (KNIES *et al*., 2012) also pointed out that single heterozygous mutation in one FA gene may be accompanied by the heterozygous mutation in other FA genes and lead to the disease. Our previous detailed molecular-cytogenetic investigation of these patients showed that cases 1 and 2 in which 7 and 8 variants were found, respectively, developed, at the time of sampling, severe BMF, had shorter telomeres, more telomere fusions and radial figures and different chromosomal breakage pattern compared to other FA-D2 patients (JOKSIC *et al*., 2012; FILIPOVIC *et al*., 2016). This is consistent with Chang's statement that great number of variants reflects the susceptibility of FA pathway.

Comparing the results obtained by Sanger sequencing and *in-silico* analysis showed that only in two patients, potentially pathogenic mutation was not detected, whereas in one patient only one pathogenic variant was found, in examined DNA regions. However, we only did screening for certain commonly affected *FANCD2* regions, so the disease-causing mutation could be in other *FANCD2* regions, or in the form of a large deletion/insertion, which could not be detected by sequencing techniques. We are aware that in examined patients' cohort, which is rather small, further analyses are required, such as next generation sequencing and whole exome sequencing (NGS and WES) for complete coverage and multiple ligation-dependent probe amplification (MLPA) and microarray techniques for the large mutations detection (GILLE *et al*., 2012; CHANG *et al*., 2014; CHANDRASEKHARAPPA *et al*., 2013). NGS and WES could provide greater coverage; however, deep intronic mutations are not detectable by WES (KNIES *et al*., 2012). Additionally, the presence of two *FANCD2* pseudogenes can lead to the incorrect mapping of the variants by NGS and WES (KNIES et al., 2012), which still makes these methods insufficiently reliable and requires confirmation by Sanger sequencing.

Summarized, we found three novel, yet unreported variants in *FANCD2* gene, two of which could be novel disease-causing mutations. Except one, all variants were present in heterozygous state, whereas the majority was found in deep intronic regions. Many of the detected variants were found in more than one patient, including those unreported, indicating their possible ethnic association. Finding of a great number of variants in some patients suggests their non-random occurrence in susceptible FA pathway.

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GENOTIPIZACIJA PACIJENATA SA FANKONIJEVOM ANEMIJOM IZ SRBIJE OTKRIVA TRI NOVE VARIJANTE GENA *FANCD2*

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Izvod

Fankonijeva anemija (FA) predstavlja retko nasledno oboljenje koje karakteriše širok spektar kongenitalnih anomalija, progresivna pancitopenija i sklonost ka razvoju hematoloških maligniteta i solidnih tumora. Molekularno genetička analiza mutacija u *FANC* genima je veoma značajna za potvrdu dijagnoze oboljenja, prenatalno i testiranje nosioca, kao i predikciju ishoda hemoterapije i komplikacija oboljenja. Ispitivali smo prisustvo varijanti u često izmenjenim regionima gena *FANCD2* (6 egzona i 5 introna) kod 6 FA-D2 pacijenata u Srbiji metodom Sangerovog sekvenciranja. Ovo je prva molekularna analiza *FANCD2* gena kod FA-D2 pacijenata u Srbiji. Detektovano je ukupno 10 varijanti, jedna homozigotna i 9 heterozigotnih, od toga dve u egzonima, a 8 u dubokim intronskim regionima. *In-silico* analiza je pokazala da od svih detektovanih varijanti jedna egzonska i tri intronske mogu uticati na "splajsing". Heterozigotne varijante u intronu 3, c.206-246delG; egzonu 26, c.2396 C>A i intronu 28, c.2715+573 C>T nisu prethodno prijavljene. *In-silico* analiza je pokazala da dve među njima (intron 3, c.206-246 delG i egzon 26, c.2396 C>A) mogu biti nove mutacije koje uzrokuju bolest. Mnoge varijante su detektovane kod više od jednog pacijenta, uključujući neprijavljene, što bi moglo ukazivati na etničku asocijaciju. Veliki broj varijanti kod nekih pacijenata ukazuje na njihovu ne-nasumičnu pojavu u putu FA.

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